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Identification, Characterization, and Application of a Recombinant Antigen for the Serological Diagnosis of Feline Hemotropic Mycoplasma Infections

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1 Summary

In felids, three hemotropic mycoplasma species (hemoplasmas) have been described: *Mycoplasma haemofelis* (Mhf), '*Candidatus* Mycoplasma haemominutum' (CMhm), and '*Candidatus* Mycoplasma turicensis' (CMt). Particularly, Mhf may cause severe, potentially life-threatening hemolytic anemia. No routine serological assays for feline hemoplasma infections are available. Thus, the goal of this project was to identify an Mhf antigen (DnaK), to be used as a recombinant antigen in serological assays for the diagnosis of feline hemoplasma infections. The gene sequence of this protein was determined using consensus primers and blood samples from naturally and experimentally Mhf infected cats, and a naturally infected Iberian lynx (*Lynx pardinus*). Mhf DnaK was expressed recombinantly in an *E. coli* DnaK knock-out strain and purified using Ni-affinity, size exclusion, and anion exchange chromatography. It was then biochemically and functionally characterized and showed characteristics typical for DnaKs (secondary structure profile, thermal denaturation, ATPase activity, and DnaK complementation). Moreover, its immunogenicity was assessed using serum samples from experimentally hemoplasma infected cats. When the protein was applied in Western blot and enzyme-linked immunosorbent assays, it was recognized by sera from cats infected with Mhf, CMhm and CMt, respectively, but not from uninfected cats. This is the first description of a full-length purified recombinant feline hemoplasma antigen.

2 Introduction

Hemotropic mycoplasmas (hemoplasmas) are small (0.3 – 0.8 µm) epierythrocytic bacteria, which have previously been known as *Haemobartonella* and *Eperythrozoon* species. In felids, *Mycoplasma haemofelis* (Mhf), '*Candidatus* Mycoplasma haemominutum' (CMhm) and '*Candidatus* Mycoplasma turicensis' (CMt) have been described (5, 6, 19, 36). They vary in their pathogenicity, responsiveness to antimicrobial drugs, and probably in their ability to form a carrier state (5, 26, 36). Particularly Mhf may cause severe, potentially life-threatening hemolytic anemia (5).

Real-time PCR assays are the tools of choice for diagnosing and differentiating feline hemoplasma infections (29, 36). However, they may not detect all hemoplasma infections, e.g. due to fluctuating Mhf bacteremia (29), reduced bacterial blood loads after antibiotic treatment (5), or chronic carrier status of infected animals with undetectable numbers of circulating hemoplasmas (28). To overcome the resulting diagnostic gap and to further characterize the course and pathogenesis of feline hemoplasma infections, a diagnostic assay based on serum antibody detection would be desirable.

To date, no routine serological assays for the diagnosis of feline hemoplasma infections are available. The development of such assays has been significantly hampered by the fact that hemoplasmas cannot be cultured *in vitro*, and therefore antigens have had to be produced by experimental infection of cats with hemoplasmas. Experimental serological assays have been described using hemoplasma antigen either on blood smears (5) or purified from large volumes of blood (1) from infected cats. Western blot (WB) analyses of *Haemobartonella felis* antigen preparations resulted in the identification of five antigens recognized by sera from experimentally *H. felis* infected cats (1). A recent study identified Mhf antigens in crude antigen preparations from erythrocytes collected from an experimentally infected cat (21). Those antigens reacted with plasma antibodies of cats collected at different time points after experimental infection, when applied in WB analyses. The first recombinant hemoplasma antigen, *Mycoplasma suis* HspA1, was developed during a study in experimentally *M. suis*

infected pigs for the application in WB and enzyme-linked immunosorbent assays (ELISA) (10). This antigen belongs to the heat shock protein 70 (HSP70) family. It was found to be DnaK-like and present on the surface of *M. suis* (10). DnaKs are molecular chaperones consisting of an N-terminal nucleotide-binding domain (ATPase activity) which generates the energy necessary to refold misfolded proteins in cell stress situations (9). Misfolded proteins bind to the C-terminal substrate-binding domain of DnaKs. Most recently, we developed a recombinant feline hemoplasma antigen to demonstrate seroconversion of experimentally CMt infected cats in preliminary WB analyses (18). The antigen described was a truncated Mhf DnaK form, recombinantly expressed in *E. coli*, which was only partially purified leading to large inter-batch variations with regard to quality and purity. The described assay did not allow for quantification of antibody levels.

The aim of this study was to identify the complete DnaK gene of Mhf, to recombinantly produce, highly purify and characterize the antigen, and to apply it in an ELISA as a quantitative serological tool for feline hemoplasma infection.

3 Materials and methods

3.1 Animals, experimental hemoplasma infections, and samples

For the present study, samples from the following six hemoplasma infected felids were used: the experimentally Mhf infected specified pathogen-free (SPF) cat QLA5 (Liberty Research; Waverly, NY/USA), the naturally Mhf infected Swiss domestic pet cats 1008 and 7415 (35), the naturally Mhf infected free living Iberian lynx (*Lynx pardinus*) Dalia (16), the experimentally CMt infected SPF cat Y (18), and the experimentally CMhm and feline leukemia virus co-infected cat 09NFR2 (Liberty Research) ((7); www.vet.uzh.ch/dissertationen/index.php). In addition to samples from cats QLA5, 09NFR2, and Y, pre and post hemoplasma infection samples from 17 SPF cats described earlier (7, 18) were used for the ELISA evaluation.

For experimental Mhf infection, the SPF cat QLA5 was inoculated intraperitoneally at the age of 2.7 years with 2 ml of DMSO-preserved (20% v/v) Mhf positive blood (10^9 copies/ml) from the experimentally infected cat HF3 (31). The inoculum had been stored at -80°C until use. The experimental CMhm infection of the SPF cat 09NFR2 with a Spanish CMhm isolate derived from an Iberian lynx has been described previously (7). All SPF cats were kept in groups (QLA5 was kept together with a female neutered SPF companion cat), examined clinically prior to the study and their SPF status was verified as described previously (18).

After hemoplasma inoculation, EDTA-anticoagulated whole blood samples were collected regularly, hemograms were generated using a Cell-Dyn 3500 (Abbott; Baar/Switzerland), and quantification of hemoplasma blood loads was performed by TaqMan real-time PCR (35). Serum or plasma samples were collected for serological analyses (see below). Anemia was defined as hematocrit value less than 33 % (equals 5 % quantile of reference range determined in our laboratory using identical methods and blood samples from 58 clinically healthy cats). EDTA-anticoagulated blood samples collected from cat QLA5 10 days post infection (DPI; 4.4 x 10^8 copies/ml blood), cat 1008 (2.8 x 10^8 copies/ml blood), cat 7415 (8.0 x 10^6 copies/ml blood), and from lynx

Dalia (6.6 x 10^4 copies/ml blood) were used for Mhf DnaK gene amplification and sequencing. DNA from 1 ml blood of cat QLA5 was extracted manually using the QIAmp DNA Blood Mini Kit (Qiagen; Hombrechtikon/Switzerland). Total nucleic acids from cats 1008, 7415 and lynx Dalia were extracted from 200 µl of blood using the MagNa Pure LC Total Nucleic Acid Isolation Kit I (Roche Diagnostics; Reinach/Switzerland).

3.2 Determination of Mhf DnaK gene sequence and phylogenetic analysis

Based upon the sequence information of Mhf DnaK gene fragments AY150993 (303 bp) and FJ463263 (899 bp) and of all other mycoplasma DnaK gene sequences available from the GenBank database until June 2009, several consensus primer pairs were designed manually and tested for the amplification of the potential Mhf DnaK gene. Using the primer pairs F1-35Mp/R934-956Mhf and F600-623Var/R1746-1768Ms (Table 1), the Mhf DnaK gene was amplified as two overlapping fragments of 976 bp and 1,307 bp length, respectively. The following thermal cycling conditions were applied: initial denaturation at 98°C for 180 s, 35 cycles of 98°C for 10 s, 60°C (976 bp) or 63°C (1,307 bp) for 30 s, 72°C for 60 s, followed by final elongation at 72°C for 10 min. All DNA amplification steps of this study were performed using Phusion High-Fidelity DNA polymerase (Finnzymes; Espoo/Finland). The resulting PCR products of expected lengths were extracted from agarose gel using the NucleoSpin Extract II kit (Macherey-Nagel; Düren/Germany) according to the manufacturer's instructions. Gene sequences of the PCR products were determined by DNA sequencing (Microsynth; Balgach/Switzerland). Resulting Mhf DnaK gene and deduced protein sequences were compared to non-redundant nucleotide and amino acid database sequences using the BLASTN and BLASTP algorithms (http://blast.ncbi.nlm.nih.gov/Blast.cgi) and were aligned to the mycoplasma DnaK sequences of species shown in Fig. 1 using ClustalW2 (11). The phylogenetic tree of mycoplasma DnaK protein sequences was constructed using MEGA version 4 (27). Bootstrap support (1,000 replicates) was calculated by the neighbor-joining method.

3.3 Gene construction and molecular cloning

The recombinant Mhf DnaK (Mhf rDnaK) gene was obtained in two steps: first, the Mhf DnaK gene was amplified as two overlapping fragments from DNA extracted from blood of cat QLA5 (primer pairs F1-35Mp/R934-956Mhf and F666-691Mhf/R1750-1783Mhf; Table 1). The resulting 976 bp and 1,119 bp long PCR products were extracted from agarose gel as described above and assembled in a second step, which combined 10 ng of each of those fragments in an overlap extension PCR (50 µl reaction volume) using the overhang primers FDnaKMhfpET and RDnaKMhfpET (Table 1). Those primers also inserted *Nde*I- and *Xho*I-cleavage sites at the 5'- and 3'-ends of the gene, respectively. The following cycling conditions were used: 5 cycles with an annealing temperature of 85°C followed by the addition of primers and 35 cycles with an annealing temperature of 60°C (remaining cycling conditions as described above). The resulting PCR product (1,824 bp) was extracted from agarose gel, digested with restriction enzymes *Nde*I and *Xho*I (New England Biolabs; Ipswich, MA/USA) according to the manufacturer's instructions and ligated to the 4,690 bp *Xho*I-*Nde*I fragment of vector pMG211 (24). The vector contained an ampicillin resistance gene, a salicylate-inducable promoter, and the genetic information for a C-terminal 6 x His tag followed by a stop codon. The correct Mhf rDnaK gene sequence within pMG211 was confirmed by DNA sequencing before protein production. For the DnaK complementation assay, the gene of *E. coli* chorismate mutase (EcCM; (12, 25)) was *Nde*I- and *Xho*I-digested and ligated to the *Xho*I-*Nde*I fragment of plasmid pMG211 as described above.

TABLE 1. Primers used for amplification and construction of the Mhf (r)DnaK gene

Primer name[a]	Sequence 5' to 3'	Length (bp)	Product length (bp)
F1-35Mp	GGCAAAAGAAATAATTTTAGGAATTGACTTAGG	33	976
R934-956Mhf	CTTAACCAACTCCTGAACGGCAG	23	
F600-623Var	GTGGTGACGATTGGGATCAAGC	22	1,307
R1746-1768Ms	CTGATGCAGCTTGTCCTCCAGCA	23	
FDnaKMhfpET	GCAGCA**CATATG**GCAAAAGAAATAATTTTAGGAA TTGACTTAGG[b]	44	1,824
RDnaKMhfpET	GCAGCA**CTCGAG**GGATTTAGTTTTATCTACCTCA GTCTTATCCTC[c]	45	
F666-691Mhf	GCACTTCAAAGACTTAAGGATGCCGC	26	1,119
R1750-1783Mhf	TTAGGATTTAGTTTTATCTACCTCAGTCTTATCC	34	

[a] Primer names indicate forward (F) and reverse (R) orientation of the primers. All primers were used at a final concentration of 0.5 μM each.

[b] Bold: *Nde*I recognition site.

[c] Bold: *Xho*I recognition site.

3.4 Prokaryotic expression and purification of recombinant Mhf DnaK

Plasmid pMG211, containing the Mhf rDnaK gene (naturally without UGA readthroughs), was transformed into the *rec*A-deficient *E. coli* strain XL1 blue (Stratagene; LaJolla, CA/USA) for plasmid storage and multiplication. Cells were grown on LB agar containing 150 µg/ml ampicillin and in LB medium containing 200 µg/ml ampicillin at 37°C and 250 rpm, respectively. Plasmid DNA was purified using Jetquick Plasmid Miniprep Spin kit (Genomed; Löhne/Germany). Transformed XL1 blue cells were stored as glycerol stocks at -80°C. For protein production, plasmid pMG211 Mhf rDnaK was transformed into the kanamycin resistant strain JW0013, an in-frame DnaK knockout mutant of *E. coli* K-12 (2). Preparative cultures were inoculated from overnight starter cultures and grown at 30°C and 250 rpm in LB medium containing 150 µg/ml ampicillin and 25 µg/ml kanamycin. Gene overexpression was induced with 1 mM salicylate at OD_{600} of 0.6, and the culture was incubated for an additional 20 hours at 25°C and 250 rpm. After cell lysis using 1 mg/ml lysozyme and ultrasonication, protein was purified from the soluble fraction by affinity chromatography on Ni^{2+}-NTA agarose (Qiagen). The purification progress was assessed by SDS-PAGE analysis (see below) after each purification step. Fractions containing monomeric Mhf rDnaK were isolated by size-exclusion chromatography on a calibrated Superdex 200 10/300 GL column (Amersham Pharmacia Biotech; Uppsala/Sweden) in TBS, pH 7.4. Those fractions were then subjected to anion exchange chromatography on a Mono Q HR 16/10 column (Amersham Pharmacia Biotech) in TBS, pH 7.4 using a salt gradient from 150 mM to 500 mM NaCl. Fractions containing protein with a molecular mass of about 66 kDa were combined, concentrated using Amicon Ultra Centrifugal Filters 10 K (Millipore; Carrigtwohill, Cork/Ireland), and their protein concentration was determined by the Bradford assay (Coomassie Plus Protein Assay Reagent, calibrated with BSA; Thermo Scientific; Rockford, IL/USA).

3.5 Molecular mass determination of recombinant Mhf DnaK

The molecular mass of Mhf rDnaK protein was determined at the protein service unit of the Functional Genomics Center Zurich (FGCZ), University of Zurich, Switzerland. The

purified protein solution was analyzed using electrospray ionization mass spectrometry. The experimentally determined molecular mass was then compared to the mass calculated by the ProtParam tool (www.expasy.ch/tools/protparam.html) based on the deduced protein sequence of Mhf rDnaK.

3.6 Structure and stability determination

Circular dichroism (CD) spectroscopy was performed on an Aviv Circular Dichroism Spectrometer Model 202 (Aviv Instruments Inc; Lakewood, NJ/USA) in quartz cuvettes of 0.2 cm pathlength (*d*). Far-UV spectra were recorded from 260 to 200 nm in 1 nm steps at 25°C and 1 µM Mhf rDnaK protein concentration (*c*) in TBS, pH 7.4 (50 mM tris base, 150 mM NaCl). For stability studies, KCl (100 mM) together with $MgCl_2$ (2.5 mM) and/or ATP (0.1 mM) were added. Data were collected for 5 s at each step. Five scans were averaged and buffer spectra determined under identical conditions were subtracted. The observed ellipticity (θ_λ) at wavelength λ was transformed into molar ellipticity per residue ($\theta_{m,r}$) using equation 1 (where *n* is the number of residues).

(1) $$\theta_{m,r} = \frac{\theta_\lambda}{10 \cdot c \cdot d \cdot n}$$

Thermal denaturation experiments were performed in TBS, pH 7.4, at 1 µM protein concentration by monitoring the CD signal at 222 nm from 10°C to 95°C and reverse in 0.5 K steps with 60 s temperature equilibration, 60 s data collection, and 1 K per minute heating/cooling rate between temperature steps. T_m, the melting point of the Mhf rDnaK ATPase domain, was defined as the inflection point of the melting curve and was determined from the first derivative after curve smoothing using the SigmaPlot v11.0 software package (Systat Software Inc; Richmond, CA/USA).

3.7 ATPase activity assay

ATPase activity was measured using a spectrophotometric assay (32), which quantified the released amount of inorganic phosphate (P_i) during ATP hydrolysis. The reaction of 2-amino-6-mercapto-7-methylpurine-ribonucleoside (MESG) with P_i was catalyzed by the purine nucleoside phosphorylase (PNP; Sigma-Aldrich; Buchs/Switzerland) and led

to a measurable change in absorbance at 360 nm. MESG was synthesized and purified as previously published (32). All measurements were performed at 25°C and 1 U/ml PNP. The change in absorbance at 360 nm was calibrated to the P_i concentration using MESG (190 µM) and P_i (0, 1, 5, 25, and 50 µM; from 200 µM Na_2HPO_4-solution). Michaelis-Menten kinetic measurements of Mhf rDnaK where then performed under the following ATPase assay conditions: 50 mM Tris, 100 mM KCl, 2.5 mM $MgCl_2$, 400 nM purified Mhf rDnaK, and 380 µM MESG. The change in absorbance at 360 nm was measured in duplicates for 26 min at ATP concentrations of 10, 50, 100, 250, and 500 µM after an equilibration time of 4 min in a Lambda 35 spectrophotometer (PerkinElmer; Waltham, MA/USA). Blank values measured without Mhf rDnaK were subtracted and the reaction rates were calculated. The catalytic parameters k_{cat} and K_m were determined from curve fitting to the Michaelis-Menten equation using the SigmaPlot v11.0 software package (Systat Software Inc).

3.8 DnaK complementation assay

The *E. coli* DnaK knock-out mutant strain JW0013 was transformed with plasmid pMG211 containing either the Mhf rDnaK or the EcCM gene (as negative control). Liquid cultures were prepared from both transformants and incubated at 30°C and 250 rpm overnight. Cell densities of both cultures were adjusted to OD_{600} of 3.0 and cultures were then diluted sequentially tenfold down to 10^{-6}. One 5 µl drop of each culture and dilution was placed on LB agar plates containing 150 µg/ml ampicillin, 25 µg/ml kanamycin and 1 mM salicylate. Agar plates were incubated without ventilation at 30°C or 43°C, respectively. Bacterial growth was assessed after 22.5 hours by counting colonies.

3.9 SDS-PAGE and recombinant antigen-based Western blot

SDS-PAGE and protein transfer to nitrocellulose membranes were performed in a PhastSystem High Speed Electrophoresis System, a PhastSystem development unit, and a PhastTransfer semi-dry electrophoretic blotting unit (all Amersham Pharmacia Biotech). Recombinant Mhf DnaK (540 ng/lane) was separated on PhastGel 20 % (w/v) homogenous SDS polyacrylamide gels (Amersham Pharmacia Biotech), transferred,

and Coomassie-stained according to the manufacturer's instructions. WBs were probed with pre and post infection serum or plasma samples from the experimentally infected cats QLA5 (Mhf; prior to and 21 DPI), 09NFR2 (CMhm; prior to and 56 DPI), and Y (CMt; prior to and 109 DPI). Serum and plasma samples were diluted 1:100 in blocking buffer (150 mM NaCl, 10 mM tris base, 20 g/l skimmed milk powder). Serum antibodies bound to Mhf rDnaK were visualized using peroxidase-conjugated, affinity-purified goat anti-cat IgG antibodies (diluted 1:2,000 in blocking buffer; Jackson ImmunoResearch Laboratories; West Grove, PA/USA) and 4-chloro-1-napthtol as the chromogenic agent.

3.10 Recombinant antigen-based ELISA

Mhf rDnaK was heated in coating buffer (100 mM Na_2CO_3, 0.1 % (w/v) SDS, pH 9.6) to 100°C for one minute and then diluted 1:20 in coating buffer without SDS. Flat-bottomed 96-well microtiter plates with medium binding capacity (Greiner Bio-One; Frickenhausen/Germany) were then coated with 100 µl/well of this Mhf rDnaK solution for three hours at 37°C and overnight at 4°C. Plates were subsequently washed three times with 200 µl/well washing buffer (150 mM NaCl, 0.05 % (v/v) Tween 20) and incubated for 1 hour at 37°C with 100 µl/well blocking buffer (150 mM NaCl, 50 mM tris base, 1 mM Titriplex III, 0.1 % (w/v) bovine serum albumin (BSA), 0.1 % (v/v) Tween 20, pH 7.4). After washing as described above, 100 µl of serum samples diluted in serum buffer (blocking buffer without BSA) were added per well and incubated for 1 hour at 37°C. The plates were washed, each well was then filled with 100 µl of peroxidase-conjugated, affinity-purified goat anti-cat IgG antibodies (diluted 1:3,000 in serum buffer; Jackson ImmunoResearch Laboratories) and incubated for 1 hour at 37°C. After washing, 100 µl/well substrate solution (150 mM citric acid pH 4.0, 1 % (v/v) H_2O_2 2 %, 1 % (v/v) 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt) were added and plates were incubated at room temperature for 10 minutes. Absorbance was then measured at a wavelength of 415 nm using a Spectramax Plus 348 microplate spectrophotometer (Molecular Devices; Sunnyvale, CA/USA).

Optimal serum dilutions and recombinant antigen concentrations were established by a checkerboard titration using pre and post infection sera from the three experimentally

infected cats QLA5 (Mhf; prior to and 21 DPI), 09NFR2C (CMhm; prior to and 56 DPI), and Y (CMt; prior to and 109 DPI) at serum dilutions of 1:50, 1:100, and 1:200. Antigen amounts of 200, 100, 50, and 10 ng/well were tested in duplicates. Wells containing only antigen without serum served as blanks, wells containing pre infection serum samples served as negative controls.

Serum samples from 20 hemoplasma free SPF cats before and after hemoplasma infection were tested using optimized conditions. For cat QLA5, samples taken during the whole course of experimental Mhf infection (0 to 800 DPI), were assayed additionally.

3.11 Statistical analyses

Statistical analyses were performed using the SigmaPlot v11.0 software package (Systat Software Inc). Correlations between ELISA antibody levels and Mhf blood loads in the experimentally Mhf infected cat QLA5 were assessed using the Spearman rank order correlation test.

3.12 GenBank accession numbers

The nucleotide and deduced amino acid sequences of Mhf DnaK have been deposited in the GenBank database (HM594280, HM594281, HM594283). Additionally, a partial CMhm DnaK sequence, acquired using identical methods, was submitted (HM594282).

4 Results

4.1 Mhf DnaK sequences

The obtained sequences of the potential Mhf DnaK gene included, according to BLASTN search results, an open reading frame (ORF) most similar to mycoplasma DnaK gene sequences (data not shown). When compared to mycoplasma DnaK complete gene sequences from the GenBank database, the highest identities with this ORF were found for *M. suis* DnaK (HspA1; 72 %) and *Mycoplasma penetrans* DnaK (69 %). Identities with the two partial Mhf DnaK gene sequences FJ463263 (899 bp) and AY150993 (303 bp) were 99 % and 97 %, respectively. Accordingly, the ORF was named Mhf DnaK gene. Further comparisons of Mhf DnaK gene sequences derived from four different Mhf infected animals resulted in three distinct Mhf DnaK gene sequences: from cats 7415 and QLA5 an identical sequence was obtained (HM594280). Sequences obtained from cat 1008 (HM594283) and lynx Dalia (HM594281) shared the same 21 silent point mutations as well as one point mutation causing a serine to glycine exchange at position 580, when compared to the 7415/QLA5 sequence. The sequence from lynx Dalia (HM594281) additionally contained a point mutation causing a proline to serine exchange at position 577. When the deduced Mhf DnaK protein sequence (cats 7415/QLA5; HM594280) was compared to mycoplasma DnaK sequences from the GenBank database using the BLASTP search algorithm, again *M. suis* (70 %) and *M. penetrans* (65 %) shared highest identities. Phylogenetic analyses of the Mhf DnaK protein sequence revealed it to cluster within the haemofelis group of the hemoplasmas, distinct from other mycoplasma groups (Fig. 1).

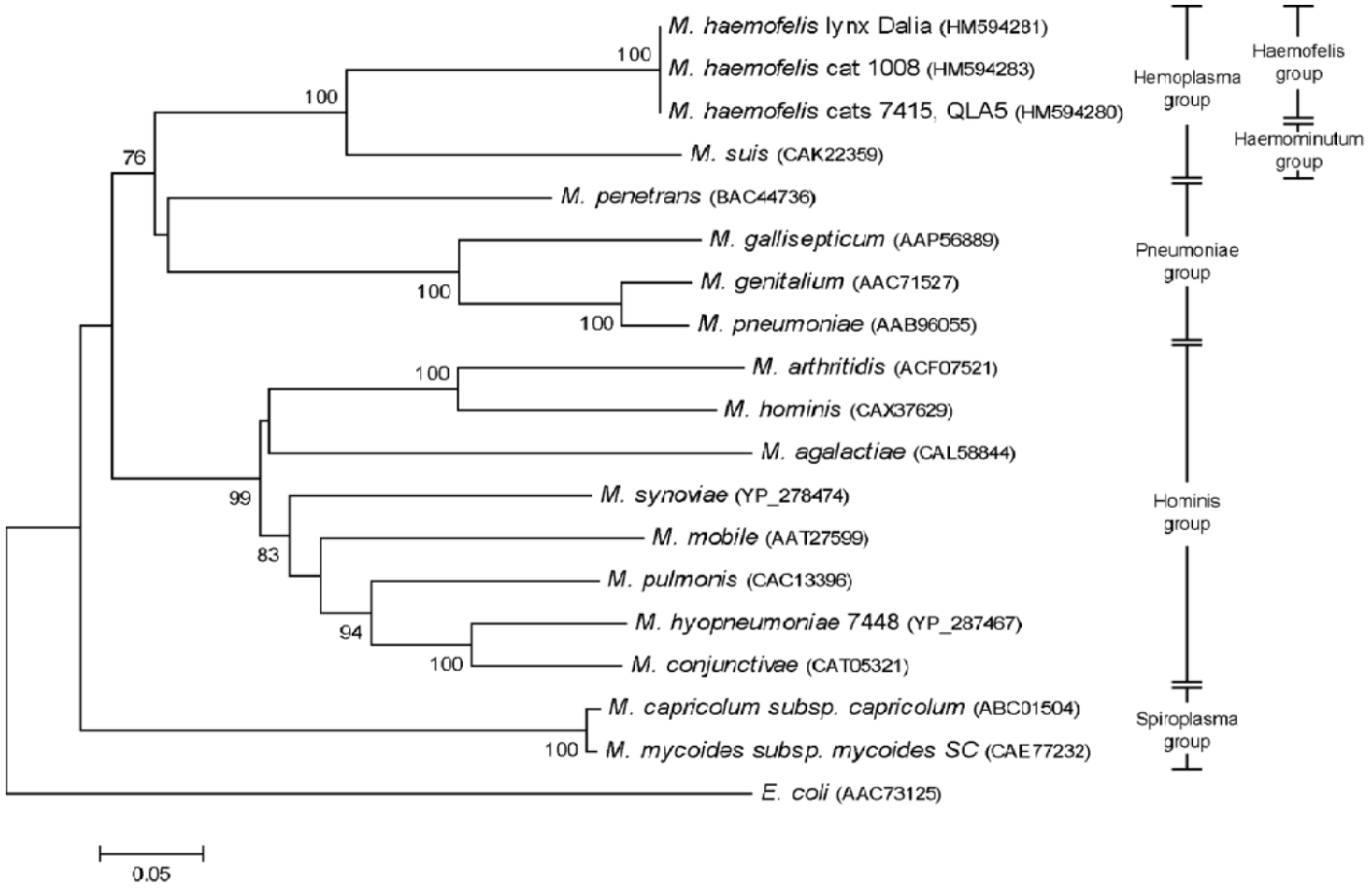

FIG. 1. Phylogenetic tree demonstrating the relationship of the deduced Mhf DnaK protein sequences to mycoplasma DnaK protein sequences from the GenBank database. Phylogenetic relationships were calculated using the neighbor joining algorithm. Evolutionary distances are shown to the scale. The data set was resampled 1,000 times to generate bootstrap percentage values. Bootstrap values greater than 70 % are given at the nodes of the tree. GenBank accession numbers are indicated in parentheses. Mycoplasma groups, which have previously been classified based on their 16S rRNA gene (23, 34) or on their RNase P RNA gene sequences (hemoplasma group, haemofelis group; (22)) are indicated. The sequence of *E. coli* served as an outgroup, establishing the root of the tree.

4.2 Expression and biochemical characterization of recombinant Mhf DnaK

Ni-affinity chromatography of crude extracts of salicylate-induced JW0013 pMG211 Mhf rDnaK cells yielded one predominant protein band corresponding to a molecular mass of about 66 kDa (data not shown). After size exclusion, anion exchange chromatography, and protein concentration, Mhf rDnaK was judged to be pure by SDS-PAGE (Fig. 3A).

Mass spectrometry analysis determined the molecular mass of Mhf DnaK to be 66,406 Da, whilst the calculated molecular mass based on its deduced protein sequence was 66,537 Da. This difference in mass of 131 Da corresponds to the N-terminal loss of methionine during mass spectrometry analysis.

CD spectrum analysis of Mhf rDnaK showed two distinct minima at 208 and 222 nm (Fig. 2A). The CD spectrum of Mhf rDnaK recorded without ATP or K^+ and Mg^{2+} ions was not markedly different from those recorded with additives. The temperature-dependent CD signals at 222 nm showed thermally induced unfolding of Mhf rDnaK with well-defined (T_{m1}) and less well-defined (T_{m2}) temperature transitions (Fig. 2B). The melting temperature of the nucleotide-binding domain of Mhf rDnaK without additives was determined as T_{m1a} of 42°C, addition of the nucleotide ATP increased it to T_{m1c} of 46°C and ATP together with K^+ and Mg^{2+} ions further increased it to T_{m1d} of 50°C. Addition of K^+ and Mg^{2+} ions alone only caused a minimal increase to T_{m1b} of 43°C.

Enzymatic activity of the ATPase domain of Mhf rDnaK was determined in a spectrophotometric assay. Fitting reaction rates at varying substrate concentrations to the Michaelis-Menten equation, yielded catalytic parameters for ATP hydrolysis (Fig. 2C).

During the DnaK complementation assay, the *in vivo* biological function of DnaKs to allow bacterial growth at cell-stressing temperatures, was tested for Mhf rDnaK. No difference in the extent of bacterial growth between the Mhf rDnaK and the EcCM transformant could be seen for any of the tested culture dilutions at 30°C (Fig. 2D). At 43°C, however, cells overexpressing EcCM and lacking DnaK protein only grew until a dilution of 10^{-1} (20 colonies), while for rDnaK expressing cells growth until a 10^{-3} dilution (1 colony) was observed (Fig. 2D).

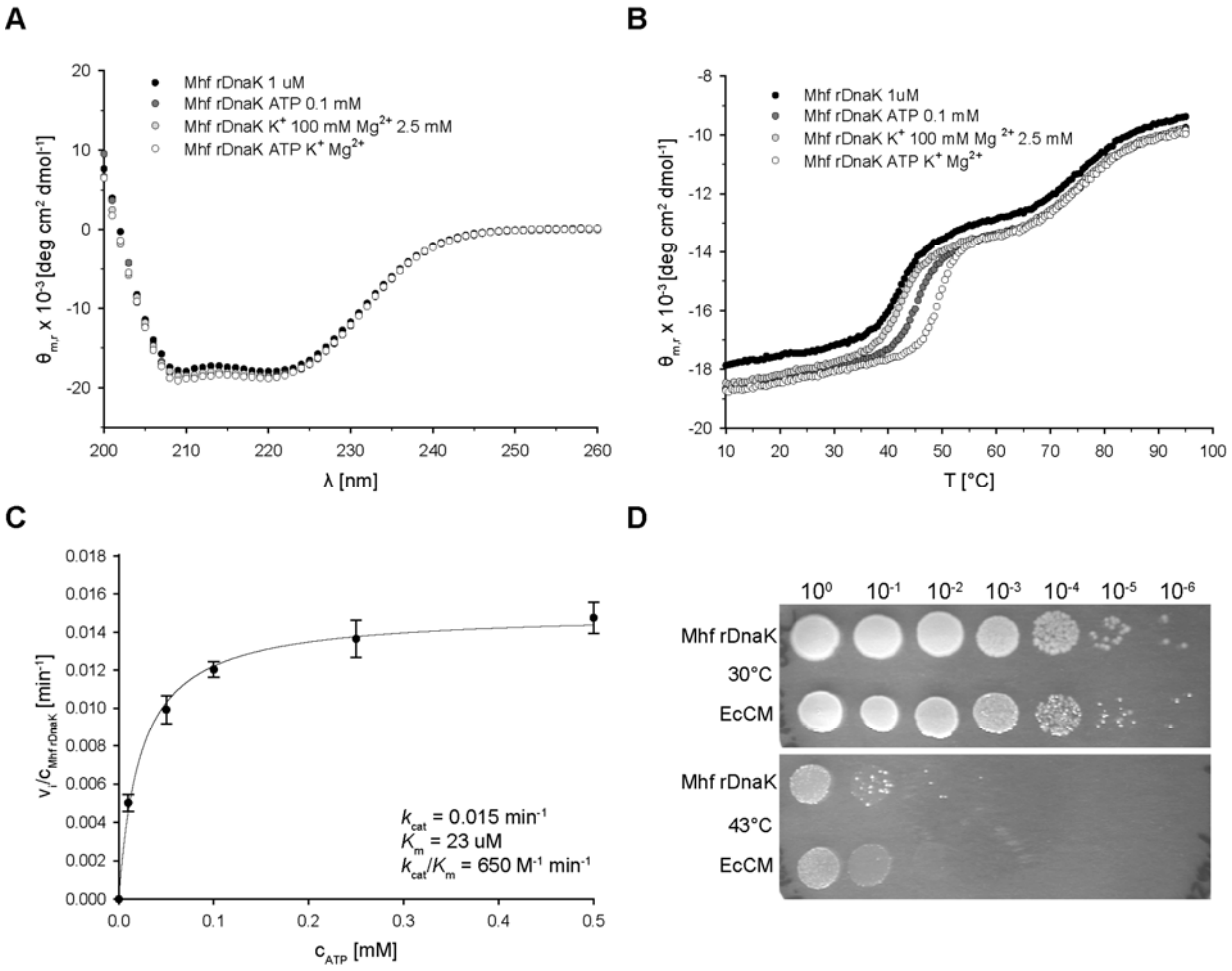

FIG. 2. Biochemical and functional characterization of Mhf rDnaK. **(A)** Circular dichroism spectra of Mhf rDnaK in the absence or presence of ATP and/or K^+ and Mg^{2+} ions. **(B)** Thermal denaturation curves of Mhf rDnaK recorded at a wavelength of 222 nm in the absence or presence of ATP and/or K^+ and Mg^{2+} ions. *T*, temperature **(C)** Michaelis-Menten plot of Mhf rDnaK (400 nM) showing ATPase activity at indicated ATP concentrations. The kinetic constants k_{cat} and K_m of Mhf rDnaK were determined from curve fitting to the Michaelis-Menten equation. **(D)** Evaluation of *in vivo* complementation of biological DnaK function by Mhf rDnaK in the DnaK-deficient *E. coli* strain JW0013 at permissive (30°C; upper panel) and non-permissive (43°C; lower panel) temperatures. JW0013 cells were either expressing the Mhf rDnaK gene (Mhf rDnaK) or the *E. coli* chorismate mutase gene (EcCM), the latter serving as negative control. Overnight cultures of transformed cells were diluted sequentially tenfold down to 10^{-6} before being spotted onto selective agar plates supplemented with 1 mM salicylate. Their ability to grow was evaluated by counting colonies.

4.3 Immunogenicity of the recombinant Mhf DnaK

WB analyses of Mhf rDnaK showed that the protein was recognized by serum antibodies from cats experimentally infected with Mhf (cat QLA5), CMhm (cat 09NFR2), and CMt (cat Y). Pre infection serum or plasma samples from the same cats did not result in a positive WB signal (Fig. 3B).

For the Mhf rDnaK ELISA, 50 ng Mhf rDnaK/well and a dilution of 1:200 for sera from Mhf infected cats and 1:100 for sera from CMhm and CMt infected cats were found to be the optimal conditions. OD_{415} values for serum samples from 20 SPF cats prior to hemoplasma infection ranged from 0.12 to 0.33 under these conditions. The ELISA signal ratio (OD_{415} value post/pre infection) of the Mhf, CMhm and CMt infected SPF cats reached a maximum of 10.4, 10.4 and 10.6, respectively. An ELISA signal ratio of at least 1.5 was considered serologically positive for anti-Mhf rDnaK antibodies.

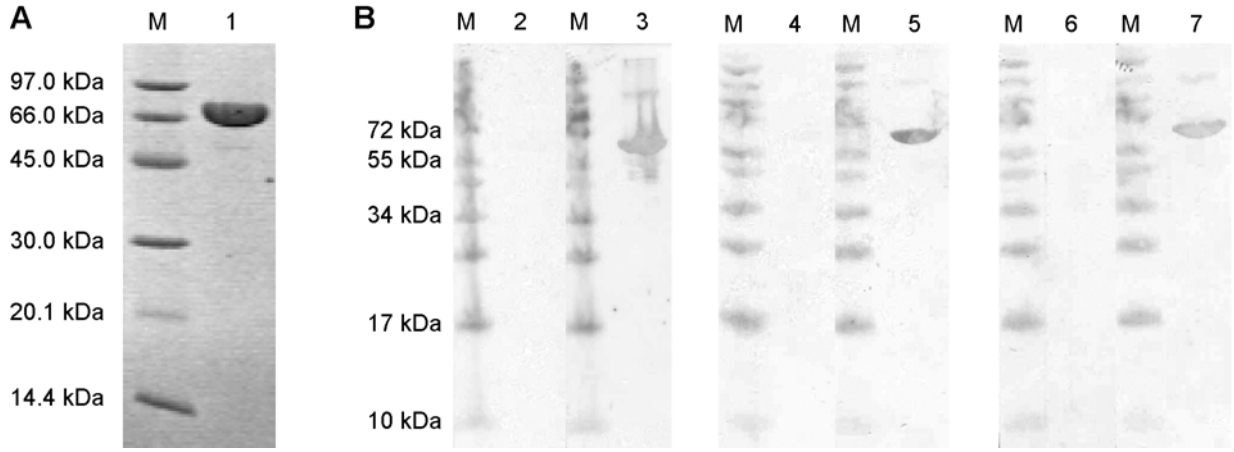

FIG. 3. (A) SDS-PAGE of recombinant Mhf DnaK for purity assessment after the final purification step of anion exchange chromatography. M, low molecular weight marker (Amersham Pharmacia Biotech); 1, Mhf rDnaK **(B)** Western blot analyses of pre and post feline hemoplasma infection samples using 540 ng recombinant Mhf DnaK per lane. Blots were reacted with serum from Mhf infected cat QLA5 prior to (lane 2) and 21 DPI (lane 3), plasma from CMhm infected cat 09NFR2 prior to (lane 4) and 56 DPI (lane 5), and serum from CMt infected cat Y prior to (lane 6) and 109 DPI (lane 7). M, peqGold prestained protein marker IV (PEQLAB Biotechnologie; Erlangen, Germany)

4.4 Experimental Mhf infection and quantification of anti-Mhf DnaK antibodies

The experimentally Mhf infected cat QLA5 turned Mhf TaqMan real-time PCR-positive within 4 DPI and became anemic within 10 DPI (Fig. 4A). On the day of infection (0 DPI) the cat was mildly anemic (hematocrit of 28 %), probably due to a baseline blood collection of 26 ml eleven days prior to Mhf infection. However, the cat recovered to hematocrit values within the reference range within a few days (7 to 9 DPI) before a decrease in the hematocrit was observed starting 10 DPI. The minimum hematocrit value of 15 % was measured 36 DPI (Fig. 4A). However, no severe clinical signs were observed that necessitated blood transfusion or antibiotic treatment during the course of infection and the cat subsequently recovered from anemia. From 148 DPI (5.3 months post infection, MPI) onwards, the hematocrit values stayed within the reference range until the end of the experiment, 28.6 MPI (Fig. 4A and data not shown).

The peak Mhf load in blood (2.2×10^8 copies/ml blood) was recorded at 29 DPI. Between 4 and 42 DPI the first marked Mhf copy number fluctuations were observed; they ranged from 10^3 to 10^8 Mhf copies/ml blood within a minimum of two days (Fig. 4A). From 3.8 to 8.3 months post infection (MPI) a second episode of copy number cycling was observed; the loads ranged from 10^2 to 10^5 copies/ml blood within a minimum of eight days. Five distinct Mhf load peaks were observed in one to two month intervals during this second cycling period. QLA5 stayed PCR-negative from 260 DPI (9.3 MPI) until the end of the observation period at 28.6 MPI (Fig. 4A and B).

Seroconversion of cat QLA5, defined as an ELISA signal ratio of at least 1.5, occurred between 8 (signal ratio 1.3) and 14 DPI (signal ratio of 6.9; Fig. 4B). QLA5 stayed serologically positive until the end of the observation period at 28.6 MPI. Twelve and eighteen MPI the ELISA signal ratio dropped to minima of 2.8, followed by a signal ratio increase to 6.0 without detectable amounts of Mhf DNA in the cat's blood (Fig. 4B). Reinfection of QLA5 by its SPF companion cat as cause for this ratio increase was excluded by negative PCR and serology results for the companion cat before, during, and at the end of the observed infection period (data not shown).

There was a significant positive correlation between the ELISA signal ratio and Mhf load when all pairs of variants throughout the whole course of infection (n = 32) were included (Spearmen correlation coefficient r = 0.619; p = 0.0002) as well as when only pairs of variants from the PCR-positive period (n = 21, r = 0.502, p = 0.0202) were included.

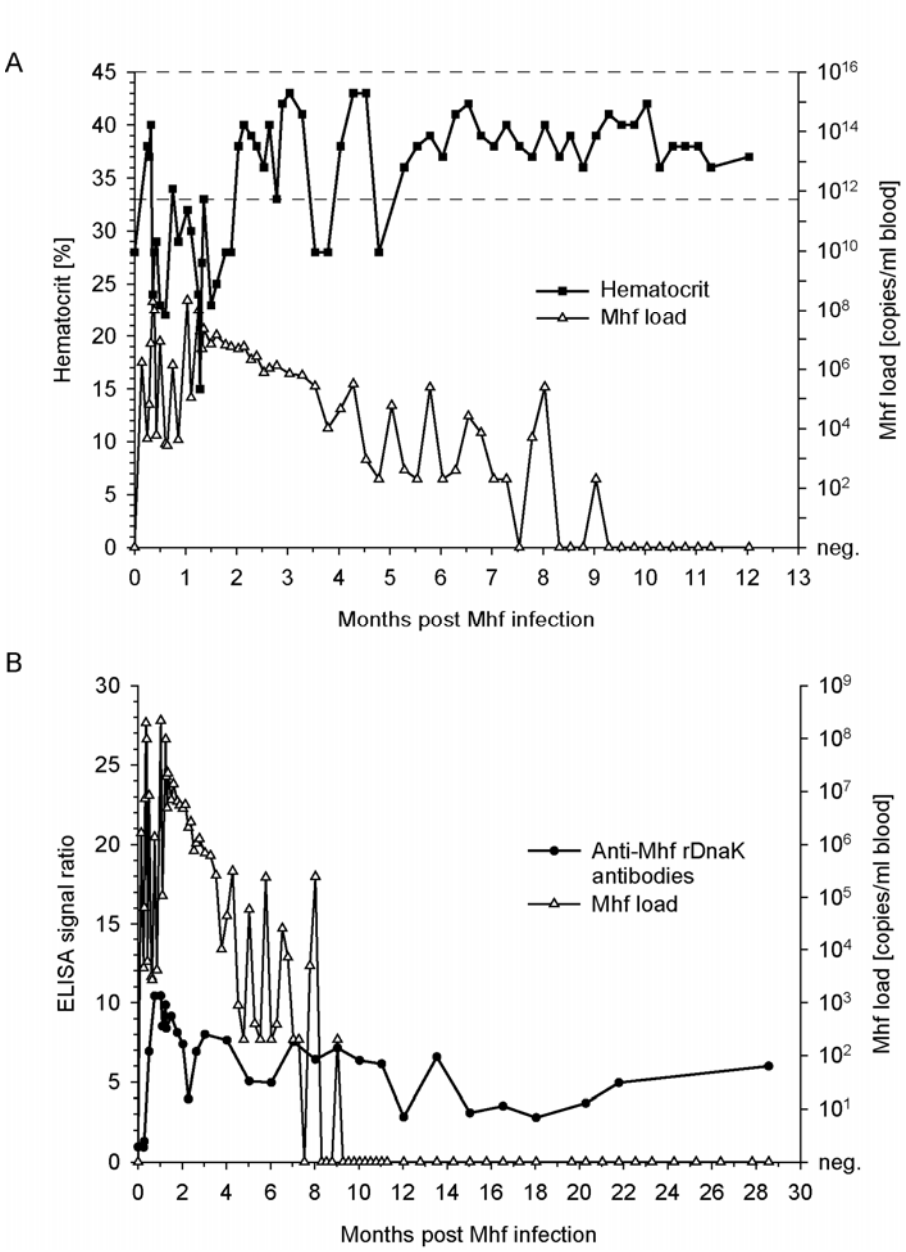

FIG. 4. Course of experimental Mhf infection in cat QLA5: hematocrit, Mhf load, and anti-Mhf rDnaK antibodies. **(A)** Hematocrit and Mhf blood copy numbers during the first year of experimental Mhf infection. Mhf blood copy numbers were determined using TaqMan real-time PCR (35). The dashed lines indicate the lower and the upper limit (5 % and 95 % quantiles) of the hematocrit reference range (33 % to 45 %). **(B)** Anti-Mhf rDnaK serum antibodies and Mhf blood copy numbers during 29 months of experimental Mhf infection.

5 Discussion

This is the first study to identify, characterize, and recombinantly produce a full-length, highly pure antigen of feline hemotropic mycoplasmas. The protein is a HSP70 and belongs to the DnaK protein family. It is most closely related to HspA1, the DnaK of *M. suis* (CAK22359); the latter protein was demonstrated to be expressed on the surface of *M. suis* cells and to have immunogenic potential (10). In analogy to this, we found that hemoplasma infected cats readily produced antibodies to Mhf rDnaK.

The protein crossreacted with sera from cats experimentally infected with Mhf, CMhm and CMt, but not with serum samples from SPF cats. However, optimization of the ELISA resulted in higher sample dilutions for Mhf samples than for CMhm and CMt samples, which possibly indicates that the immunogenicity of Mhf rDnaK is caused by conserved as well as species-specific epitopes of this antigen. This would on the one hand be in agreement with the high identity (71 %) that we found between the Mhf DnaK and the partial CMhm DnaK gene sequence (1,304 bp; HM594282) but could also explain why we were unable to amplify the 3'-end of the CMhm DnaK gene sequence using consensus primers despite several attempts (data not shown). The observed crossreactivity is also in agreement with a previous study using whole feline hemoplasma antigen preparations (5). In the latter study, antigen derived from *H. felis* large form (today known as Mhf) was tested with sera from cats infected with Mhf and CMhm (formerly known as *H. felis* small form). Mhf-derived whole hemoplasma antigen crossreacted with sera from Mhf and CMhm infected cats, while CMhm-derived antigen was only recognized by sera from CMhm infected cats.

The antigen Mhf rDnaK was purified to homogeneity from potentially antigenic proteins originating from the production process to improve the signal quality of the serological assays and to minimize inter-batch variations in antigen quality. Indeed, Mhf rDnaK protein expression and purification was repeated with identical results. The identity of the protein was proven by the comparison of the observed and calculated molecular masses of Mhf rDnaK. CD spectrum analysis of Mhf rDnaK revealed two minima (at 208 nm and 222 nm) suggesting that it consisted mostly of α-helices (8), which is in

good agreement with known DnaK structures e.g., of *E. coli* (PDB ID: 2KH0) and *G. kaustophilus* (PDB ID: 2V7Y). The structure profile of Mhf rDnaK was insensitive to change in the presence of nucleotide as has been shown before for *Bacillus licheniformis* DnaK (13) and also in the presence of K^+ and Mg^{2+} ions. Thermal denaturation of Mhf rDnaK was characterized by two temperature transitions. This corresponded well to an earlier study (17), where deletion mutants of *E. coli* DnaK were used. The authors demonstrated that the first transition (T_{m1}) was related to the unfolding of the DnaK N-terminal nucleotide-binding domain, while the second transition was related to the unfolding of the C-terminal substrate-binding domain. A raising of T_{m1} in the presence of nucleotide, as also observed for Mhf rDnaK, was reported for *E. coli* DnaK to be caused by a stabilizing effect occurring due to the ligand binding to the nucleotide-binding domain of the protein (20). As found for Mhf rDnaK, this stability was supposed to be further enhanced in the presence of nucleotide together with K^+ and Mg^{2+} ions, which mediate contacts between DnaK and nucleotide (15, 33). The kinetic constants for ATP hydrolysis by pure Mhf rDnaK (k_{cat} = 0.015/min, K_m = 23 µM, k_{cat}/K_m = 650/M/min; Fig. 2C) were comparable to those published for *E. coli* DnaK: k_{cat} values ranging from 0.02 to 0.2/min (3) and K_m values ranging from 20 nM to 20 µM (4, 14). This indicated that Mhf rDnaK possesses a typically low ATPase activity when evaluated without its cochaperones DnaJ and grPE. The DnaK complementation assay confirmed the molecular chaperone activity of Mhf rDnaK in an *E. coli* DnaK knock-out mutant at heat shock temperatures.

We demonstrated for the first time that an experimentally Mhf infected cat mounted antibodies to Mhf rDnaK within 8 to 14 days after experimental infection and shortly after the cat had turned Mhf PCR-positive in the blood. Moreover, we found a correlation between the Mhf blood loads and antibody levels. This indicates that the Mhf DnaK is immunogenic and that the recombinant antigen is suited for use in quantitative serological assays and to demonstrate seroconversion in infected animals.

The experimentally Mhf infected cat stayed serologically positive for more than two years post infection and for more than one and a half years after turning PCR-negative for Mhf in the blood. So far, we have data from only Mhf infected cat. However, earlier

results from CMt infection (18) and preliminary follow-up data from these cats (Novacco et al.; in preparation) confirm the persistence of anti-Mhf rDnaK antibodies in the absence of PCR-positivity in blood. This might indicate that there is active antigen stimulation in the chronic phase of hemoplasma infection possibly by antigen sequestered in the tissue. We have postulated that the decline of WB signal in two cats after antibiotic treatment of experimental CMt infection could have been due to therapy-induced CMt clearance from blood and tissues (18), whereas CMt antigen sequestered in the tissues of ten untreated cats could have resulted in the continuous low-level stimulation of the humoral immune system (18). This latter would be in agreement with the findings from two experimentally CMt infected cats: in nearly all tissues collected from those two cats at 20 and 94 DPI, respectively, more CMt copies were found than expected to result from blood contamination alone, which suggested that CMt might well become concentrated in organs of infected cats (personal communication S. Tasker). However, for Mhf no evidence for significant tissue sequestration has been found so far (30).

In conclusion, this study provides evidence that Mhf rDnaK, the antigen we have identified, recombinantly produced, and characterized herein, has biochemical and molecular chaperone properties of the HSP70/DnaK family. It has been applied successfully to quantify anti-feline hemoplasma antibodies in samples from experimentally infected cats, as well as to monitor seroconversion after infection when used in WB assays and ELISA. The antigen and the assays are prerequisites to gain more insight into the course of hemoplasma infections, e.g. by investigating hemoplasma pathogenesis in experimental infection setups. In addition, the described antigen may have the potential to be used in a rapid test for clinicians, supporting quick diagnosis and faster choice of adequate therapy of feline hemolytic anemia. However, further studies will be necessary to fully evaluate the crossreactivity of this antigen with antibodies directed against other bacterial pathogens and its potential benefit for testing samples from naturally hemoplasma infected cats.

6 References

1. **Alleman, A. R., M. G. Pate, J. W. Harvey, J. M. Gaskin, and A. F. Barbet.** 1999. Western immunoblot analysis of the antigens of *Haemobartonella felis* with sera from experimentally infected cats. J. Clin. Microbiol. **37:**1474-1479.

2. **Baba, T., T. Ara, M. Hasegawa, Y. Takai, Y. Okumura, M. Baba, K. A. Datsenko, M. Tomita, B. L. Wanner, and H. Mori.** 2006. Construction of *Escherichia coli* K-12 in-frame, single-gene knockout mutants: the Keio collection. Mol. Syst. Biol. **2:**1-11.

3. **Bukau, B., and A. L. Horwich.** 1998. The Hsp70 and Hsp60 chaperone machines. Cell **92:**351-366.

4. **Burkholder, W. F., C. A. Panagiotidis, S. J. Silverstein, A. Cegielska, M. E. Gottesman, and G. A. Gaitanaris.** 1994. Isolation and characterization of an *Escherichia coli* DnaK mutant with impaired ATPase activity. J. Mol. Biol. **242:**364-377.

5. **Foley, J. E., S. Harrus, A. Poland, B. Chomel, and N. C. Pedersen.** 1998. Molecular, clinical, and pathologic comparison of two distinct strains of *Haemobartonella felis* in domestic cats. Am. J. Vet. Res. **59:**1581-1588.

6. **Foley, J. E., and N. C. Pedersen.** 2001. '*Candidatus* Mycoplasma haemominutum', a low-virulence epierythrocytic parasite of cats. Int. J. Syst. Evol. Microbiol. **51:**815-817.

7. **Geret, C. P.** 2010. Disease potential of feline leukemia virus (FeLV) collected from Iberian lynxes (*Lynx pardinus*): low pathogenicity in experimentally infected specified pathogen-free domestic cats. Inaugural thesis. University of Zurich, Zurich.

8. **Greenfield, N., and G. D. Fasman.** 1969. Computed circular dichroism spectra for the evaluation of protein conformation. Biochemistry (Mosc.) **8:**4108-4116.

9. **Hartl, F. U., and M. Hayer-Hartl.** 2009. Converging concepts of protein folding *in vitro* and *in vivo*. Nat. Struct. Mol. Biol. **16:**574-581.

10. **Hoelzle, L. E., K. Hoelzle, A. Harder, M. Ritzmann, H. Aupperle, H. A. Schoon, K. Heinritzi, and M. M. Wittenbrink.** 2007. First identification and functional characterization of an immunogenic protein in unculturable haemotrophic Mycoplasmas (*Mycoplasma suis* HspA1). FEMS Immunol. Med. Microbiol. **49:**215-223.

11. **Larkin, M. A., G. Blackshields, N. P. Brown, R. Chenna, P. A. McGettigan, H. McWilliam, F. Valentin, I. M. Wallace, A. Wilm, R. Lopez, J. D. Thompson, T. J. Gibson, and D. G. Higgins.** 2007. Clustal W and clustal X version 2.0. Bioinformatics **23:**2947-2948.

12. **Lee, A. Y., P. A. Karplus, B. Ganem, and J. Clardy.** 1995. Atomic structure of the buried catalytic pocket of *Escherichia coli* chorismate mutase. J. Am. Chem. Soc. **117:**3627-3628.

13. **Liang, W. C., X. H. Wang, M. G. Lin, and L. L. Lin.** 2009. A 70-kDa molecular chaperone, DnaK, from the industrial bacterium *Bacillus licheniformis*: gene cloning, purification and molecular characterization of the recombinant protein. Indian J Microbiol **49:**151-160.

14. **Liberek, K., J. Marszalek, D. Ang, C. Georgopoulos, and M. Zylicz.** 1991. *Escherichia coli* DnaJ and GrpE heat shock proteins jointly stimulate ATPase activity of DnaK. Proc. Natl. Acad. Sci. U. S. A. **88:**2874-2878.

15. **Mayer, M. P., D. Brehmer, C. S. Gassler, and B. Bukau.** 2001. Hsp70 chaperone machines. Adv. Protein Chem. **59:**1-44.

16. **Meli, M. L., V. Cattori, F. Martinez, G. Lopez, A. Vargas, M. A. Simon, I. Zorrilla, A. Munoz, F. Palomares, J. V. Lopez-Bao, J. Pastor, R. Tandon, B. Willi, R. Hofmann-Lehmann, and H. Lutz.** 2009. Feline leukemia virus and other pathogens as important threats to the survival of the critically endangered Iberian lynx (*Lynx pardinus*). PLoS ONE **4:** doi: 10.1371/journal.pone.0004744.

17. **Montgomery, D. L., R. I. Morimoto, and L. M. Gierasch.** 1999. Mutations in the substrate binding domain of the *Escherichia coli* 70 kDa molecular chaperone, DnaK, which alter substrate affinity or interdomain coupling. J. Mol. Biol. **286:**915-932.

18. **Museux, K., F. Boretti, B. Willi, B. Riond, K. Hoelzle, L. E. Hoelzle, M. M. Wittenbrink, S. Tasker, N. Wengi, C. Reusch, H. Lutz, and R. Hofmann-Lehmann.** 2009. *In vivo* transmission studies of '*Candidatus* Mycoplasma turicensis' in the domestic cat. Vet. Res. **40:**45-58.

19. **Neimark, H., K. E. Johansson, Y. Rikihisa, and J. G. Tully.** 2001. Proposal to transfer some members of the genera *Haemobartonella* and *Eperythrozoon* to the genus *Mycoplasma* with descriptions of '*Candidatus* Mycoplasma haemofelis', '*Candidatus* Mycoplasma haemomuris', '*Candidatus* Mycoplasma haemosuis' and '*Candidatus* Mycoplasma wenyonii'. Int. J. Syst. Evol. Microbiol. **51:**891-899.

20. **Palleros, D. R., K. L. Reid, J. S. McCarty, G. C. Walker, and A. L. Fink.** 1992. DnaK, hsp73, and their molten globules. J. Biol. Chem. **267:**5279-5285.

21. **Peters, I. R., C. R. Helps, T. J. Gruffydd-Jones, M. J. Day, and S. Tasker.** 2010. Antigen specificity of the humoral immune response to *Mycoplasma haemofelis* infection. Clin Vaccine Immunol**:** doi: CVI.00136-10 [pii] 10.1128/CVI.00136-10.

22. **Peters, I. R., C. R. Helps, L. McAuliffe, H. Neimark, M. R. Lappin, T. J. Gruffydd-Jones, M. J. Day, L. E. Hoelzle, B. Willi, M. Meli, R. Hofmann-Lehmann, and S. Tasker.** 2008. RNase P RNA gene (*rnpB*) phylogeny of hemoplasmas and other *Mycoplasma* species. J. Clin. Microbiol. **46:**1873-1877.

23. **Pettersson, B., J. G. Tully, G. Bolske, and K. E. Johansson.** 2000. Updated phylogenetic description of the *Mycoplasma hominis* cluster (Weisburg *et al.* 1989) based on 16S rDNA sequences. Int. J. Syst. Evol. Microbiol. **50:**291-301.

24. **Sasso, S., C. Ramakrishnan, M. Gamper, D. Hilvert, and P. Kast.** 2005. Characterization of the secreted chorismate mutase from the pathogen *Mycobacterium tuberculosis*. FEBS J. **272:**375-389.

25. **Stewart, J., D. B. Wilson, and B. Ganem.** 1990. A genetically engineered monofunctional chorismate mutase. J. Am. Chem. Soc. **112:**4582-4584.

26. **Sykes, J. E., N. L. Drazenovich, L. M. Ball, and C. M. Leutenegger.** 2007. Use of conventional and real-time polymerase chain reaction to determine the epidemiology of hemoplasma infections in anemic and nonanemic cats. J. Vet. Intern. Med. **21:**685-693.

27. **Tamura, K., J. Dudley, M. Nei, and S. Kumar.** 2007. MEGA4: Molecular evolutionary genetics analysis (MEGA) software version 4.0. Mol. Biol. Evol. **24:**1596-1599.

28. **Tasker, S., S. M. A. Caney, M. J. Day, R. S. Dean, C. R. Helps, T. G. Knowles, P. J. P. Lait, M. D. G. Pinches, and T. J. Gruffydd-Jones.** 2006. Effect of chronic FIV infection, and efficacy of marbofloxacin treatment, on *Mycoplasma haemofelis* infection. Vet. Microbiol. **117:**169-179.

29. **Tasker, S., C. R. Helps, M. J. Day, T. J. Gruffydd-Jones, and D. A. Harbour.** 2003. Use of real-time PCR to detect and quantify *Mycoplasma haemofelis* and "*Candidatus* mycoplasma haemominutum" DNA. J. Clin. Microbiol. **41:**439-441.

30. **Tasker, S., I. R. Peters, M. J. Day, B. Willi, R. Hofmann-Lehmann, T. J. Gruffydd-Jones, and C. R. Helps.** 2009. Distribution of *Mycoplasma haemofelis* in blood and tissues following experimental infection. Microb. Pathog. **47:**334-340.

31. **Tasker, S., I. R. Peters, K. Papasouliotis, S. M. Cue, B. Willi, R. Hofmann-Lehmann, T. J. G. Jones, T. G. Knowles, M. J. Day, and C. R. Helps.** 2009. Description of outcomes of experimental infection with feline haemoplasmas: copy numbers, haematology, Coombs' testing and blood glucose concentrations. Vet. Microbiol. **139:**323-332.

32. **Webb, M. R.** 1992. A continuous spectrophotometric assay for inorganic phosphate and for measuring phosphate release kinetics in biological systems. Proc. Natl. Acad. Sci. U. S. A. **89:**4884-4887.

33. **Wegele, H., L. Muller, and J. Buchner.** 2004. Hsp70 and Hsp90 - a relay team for protein folding. Rev. Physiol. Biochem. Pharmacol. **151:**1-44.

34. **Weisburg, W. G., J. G. Tully, D. L. Rose, J. P. Petzel, H. Oyaizu, D. Yang, L. Mandelco, J. Sechrest, T. G. Lawrence, J. Vanetten, J. Maniloff, and C. R. Woese.** 1989. A phylogenetic analysis of the mycoplasmas: basis for their classification. J. Bacteriol. **171:**6455-6467.

35. **Willi, B., F. S. Boretti, C. Baumgartner, S. Tasker, B. Wenger, V. Cattori, M. L. Meli, C. E. Reusch, H. Lutz, and R. Hofmann-Lehmann.** 2006. Prevalence, risk factor analysis, and follow-up of infections caused by three feline hemoplasma species in cats in Switzerland. J. Clin. Microbiol. **44:**961-969.

36. **Willi, B., F. S. Boretti, V. Cattori, S. Tasker, M. L. Meli, C. Reusch, H. Lutz, and R. Hofmann-Lehmann.** 2005. Identification, molecular characterization, and experimental transmission of a new hemoplasma isolate from a cat with hemolytic anemia in Switzerland. J. Clin. Microbiol. **43:**2581-2585.

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